Glutathione S-Transferases in Human and Rodent Skin: Multiple Forms and Species-Specific Expression

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The glutathione S-transferases (GST) are a family of widely distributed multifunctional detoxification enzymes that catalyze the reaction between reduced glutathione and a variety of electrophiles. Of interest is the fact that several extracutaneous tissues exhibit a distinct spectrum of isozymes that are expressed in a highly controlled fashion. Despite the fact that the skin is continuously exposed to numerous injurious agents, little is known about the expression of GST isozymes and their role in metabolism of physiologic and xenobiotic substrates in cutaneous tissue. Using specific polyclonal antibodies to the Alpha, Mu, and Pi classes of GST, we identified their expression in rat, mouse, and human skin cytosol. In each species, GST isozymes expressed activities towards 1-chloro-2,4-dinitrobenzene, benzo(a)pyrene 4,5-oxide, styrene 7,8-oxide, leukotriene A_4, and ethacrynic acid, but not towards bromosulfophthalein and cumene hydroperoxide. Western blot analysis indicated the predominant expression of Pi isozyme in all three species. Alpha class of isozyme(s) was present only in human skin, whereas Mu class of isozyme(s) was detected only in rat and mouse skin. Similarly, in normal and transformed cultured human keratinocytes Pi was the predominant isozyme. In situ localization studies using immunohistochemical techniques confirmed the observations of Western blotting. In mouse skin, Pi and Mu isozyme(s) were found to be predominantly localized in sebaceous glands, whereas no reactivity was observed with the Alpha class of isozymes. Our data show that multiple forms of GST exist in rodent and human skin and that GST Pi is the predominant isozyme in each species. Furthermore, cutaneous GST can metabolize both endogenous substrates and foreign compounds. *J Invest Dermatol 96:463–467, 1991*

The glutathione S-transferases (GST) (EC 2.5.1.18), a family of multifunctional enzymes, catalyze the conjugation of a variety of exogenous and endogenous electrophilic compounds [1,2] with reduced glutathione (GSH). GST exists in multiple forms in the cytosol of various organisms [3–5], and may function as binding proteins for hydrophobic nonsubstrate ligands [6,7]. GST are also thought to play an important role in affording cellular resistance to chemotherapeutic drugs and preventing toxic injury in a variety of tissues [1–4]. Several human, rat, and mouse tissues exhibit a distinct spectrum of the GST isozyme(s) [2]. However, the interrelationship among them is not clearly understood.

Well characterized mammalian GST can be divided into three distinct species-independent classes, named Alpha (basic), Mu (neutral), and Pi (acidic) [2]. This classification of GST is based on several criteria, including substrate specificities, inhibition of catalytic activities, reactions with specific antibodies, and primary protein structure homologies. The cytosolic GST, isolated and characterized to date, are dimeric proteins; a nomenclature reflecting the subunit composition has been adapted [8]. The expression of rat GST has been shown to be tissue specific [9,10]. Studies of GST in rat testis, lung, kidney, and small intestine have revealed marked differences in the distribution of the multiple forms [10,11]. For example, GST 7-7 (Pi class) is one of the major isozymes found in lung, kidney, and small intestine. Testis contain only trace amounts of GST 7-7 and the concentration in normal liver is below the limits of detection. Similarly, GST 6-6 (Mu class), which is the most abundant form in testis, has been identified only in low concentrations in other organs [11].

Despite the fact that the skin is continuously exposed to numerous injurious agents, little is known about the expression of GST isozymes and their role in metabolism of physiologic and xenobiotic substrates in cutaneous tissue [12,13]. In the present study, we have demonstrated that multiple forms of GST exist in mammalian skin and that among these Pi is the predominant isozyme.

MATERIALS AND METHODS

Reagents and Supplies 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, GSH, and leukotriene A_4 (LTA_4)-methyl ester were obtained from Sigma Chemical Co. (St. Louis, MO). Styrene 7,8-oxide was a product of Eastman Organic Chemicals, Rochester, NY. Acrylamide and bisacrylamide were purchased from Bio-Rad Laboratory (Richmond, CA). [^{3}H]-LTA_4-methyl ester (30 Ci/mmol) and styrene oxide-8-^{14}C (0.497 mCi/mmol) were purchased

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Abbreviations:

4,5-BPO: benzo(a)pyrene 4,5-oxide
CDNB: 1-chloro-2,4-dinitrobenzene
LTA_4: leukotriene A_4
GSH: reduced glutathione
GST: glutathione S-transferase

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Table I. GST Activity in Rodent and Human Skin Cytosol[a]

Substrate	Rat	Mouse	Human
1-Choro 2,4-dinitro-benzene[b]	59.50 ± 3.91	54.07 ± 4.52	25.82 ± 1.95
Leukotriene A_4[c]	27.33 ± 2.80	12.32 ± 1.13	5.90 ± 0.42
Ethacrynic acid[b]	3.05 ± 0.22	15.50 ± 1.50	5.02 ± 0.41
Benzo(a)pyrene 4,5-oxide[a]	0.90 ± 0.05	1.22 ± 0.09	0.85 ± 0.06
Styrene 7,8-oxide[b]	4.80 ± 0.33	5.31 ± 0.33	3.91 ± 0.28
Bromosulfophthalein	Below the limits of detection		
Cumene hydroperoxide[d]	Below the limits of detection		

[a] GST activity towards various substrates was determined as described in *Materials and Methods*. Each value represents the mean ± SD of at least three samples assayed in duplicate.
[b] nmol/min/mg.
[c] pmol/min/mg.
[d] Glutathione peroxidase activity determined at 25°C.

from New England Nuclear Corp. (Boston, MA). Benzo(a)pyrene 4,5-oxide (4,5-BPO) ^{3}H (10 mCi/mmol) and unlabeled 4,5-BPO were purchased from Midwest Research Institute, Kansas City, MO. Nitrocellulose membrane for the Western blot analysis was from Schleicher and Schuell Corp. (Keene, NH). Reagents for the Western blot analysis were purchased from Promega Biotech Corp (Madison, WI). Vectastain ABC kit for immunohistochemistry was purchased from Vector Laboratories, Inc. (Burlington, CA).

Antibodies Polyclonal antibodies to purified Alpha, Mu, and Pi classes of human GST were developed in New Zealand rabbits and were the same as those used earlier [14].

Animal Treatment and Preparation of Cytosol Male Sprague-Dawley rats (150–250 g) were obtained from Holtzman Rat Farm (Madison, WI). Female SENCAR mice (25–30 g) were obtained from NCl, Frederick, MD. Dorsal skin (2 inches wide) from rat and mouse was shaved with electric clippers; hairs were removed by topical application of Nair. Skin was washed in running tap water

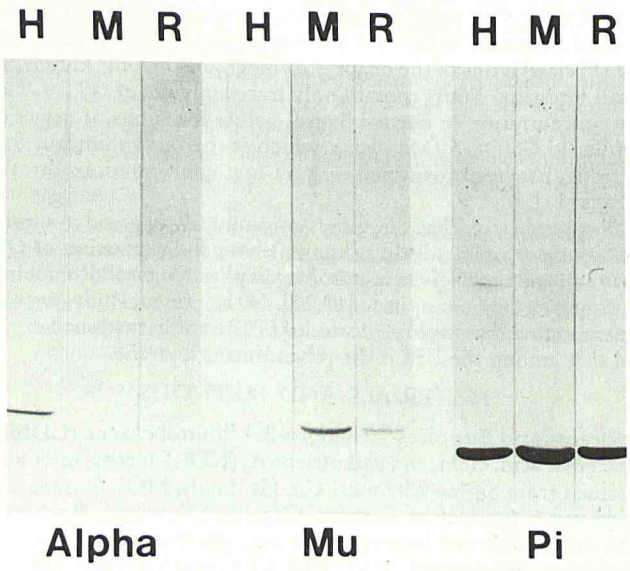

Figure 1. Western blot analysis of human and rodent skin cytosol. Skin cytosolic protein (70 μg) from human (H), mouse (M), and rat (R) was subjected to 12% SDS-PAGE and transferred onto nitrocellulose membrane by Western blotting as described in *Materials and Methods*. The membrane was probed with human polyclonal rabbit antibodies to GST (1 : 1000 dilution) and color was visualized by alkaline-phosphatase–conjugated secondary antibody.

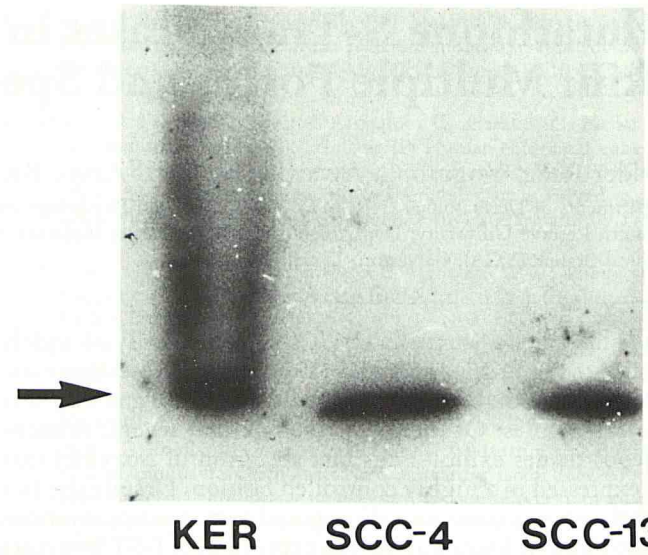

Figure 2. Western blot analysis of normal and transformed human surface epithelial cells. Equivalent amounts of total cell extract from normal keratinocytes (KER), and two squamous cell carcinoma cell lines (SCC-4, SCC-13) were electrophoresed, blotted to nitrocellulose, and incubated with a polyclonal rabbit antibody specific for GST-Pi. Binding of the primary antibody was visualized using ^{125}I-protein A followed by exposure on x-ray film for 4 d. *Arrow,* migration of the authentic GST-Pi band.

to remove excess Nair. Clean skin was dissected out and scraped with a scalpel blade to remove dermal fat, homogenized in 100 mM potassium phosphate (pH 7.4) containing 1.15 mM KCl, and the 100,000 × *g* supernatant was prepared as described previously [15].

Human Skin Human skin samples were obtained from elective abdominoplasties or breast reduction patients from the tissue conservation core facility at the Institute of Pathology, Case Western Reserve University. These specimens were collected within 2 h of surgery and appeared normal by morphologic criteria. Skin was dissected free from the adipose tissue and homogenized to prepare the 100,000 × *g* supernatant fraction as described above.

Human Keratinocyte Cell Culture Normal human epidermal keratinocytes were cultured using 3T3 feeder layer support as described by Rheinwald and Green [16]. The feeder cells were removed by treatment with EDTA prior to harvest of the epithelial cells for assay of GST. Squamous cell carcinoma cells were cultured in DMEM/Ham's F12 (3 : 1) containing nonessential amino acids, adenine (1.8×10^{-4}M), penicillin (100 U/ml), streptomycin (100

Table II. Subcellular Localization of GST Activity with CDNB as Substrate in Rat Skin[a]

Subcellular Fraction	Specific Activity (nmol conjugate/min/mg protein)
800 × *g* pellet	12.20 ± 0.81
100,000 × *g* supernatant	64.53 ± 5.32
100,000 × *g* pellet	5.35 ± 0.92

[a] Each value represents the mean ± SD of three samples.

Table III. GST Activity with CDNB as Substrate in Cytosols Prepared from Whole Skin, Dermis, and Epidermis[a]

Skin Layers	Specific Activity[b] (nmol conjugate/min/mg protein)
Epidermis	27.59 ± 2.83
Dermis	44.25 ± 3.00
Whole skin	48.32 ± 3.05

[a] 100,000 × *g* supernatant fraction from epidermis, dermis, and whole skin of 5-d-old rats was prepared as described in *Materials and Methods* and GST activity with CDNB as a substrate was measured.

[b] Each value represents the mean ± SD of four determinations.

mg/ml), gentamicin (50 ng/ml), L-glutamine (2 mM), 8% fetal calf serum, hydrocortisone (4 μg/ml), and insulin (5 μg/ml).

Enzyme Activity GST activity towards CDNB, ethacrynic acid, and bromosulfophthalein as substrate was measured according to the method of Habig et al [17]. The GST activity using 4,5-BPO and styrene 7,8-oxide as substrates was determined as described earlier [18]. The GST activity with cumene hydroperoxide was assayed as described by Lawrence and Burk [19]. Enzyme activity towards [^{3}H]LTA_4 methyl ester as substrate was determined by measuring leukotriene C_4 monomethyl ester formation as described previously by Mannervik et al [4]. Protein was estimated according to the method of Bradford [20].

Polyacrylamide Gel Electrophoresis and Western Blot Analysis For identification of GST isozymes in mammalian skin, cytosolic proteins were subjected to SDS-PAGE on 12% slab gels according to the procedure of Laemmli [21]. Resolved proteins were electrophoretically transferred onto nitrocellulose membrane by Western blotting as described by Towbin et al [22]. Specific antibody reactivity with cytosolic proteins was assessed using alkaline phosphatase conjugated anti-rabbit secondary antibody as described before [23]. For identification of GST isozyme in normal and transformed human epithelial cells, equivalent amounts of total cell extracts were electrophoresed and blotted as above. Binding of the primary antibody was visualized using ^{125}I-protein A followed by exposure on x-ray film for 4 d as described earlier [24].

Immunohistochemistry Dorsal skin from SENCAR mouse was removed and fixed in buffered 10% formalin and processed in

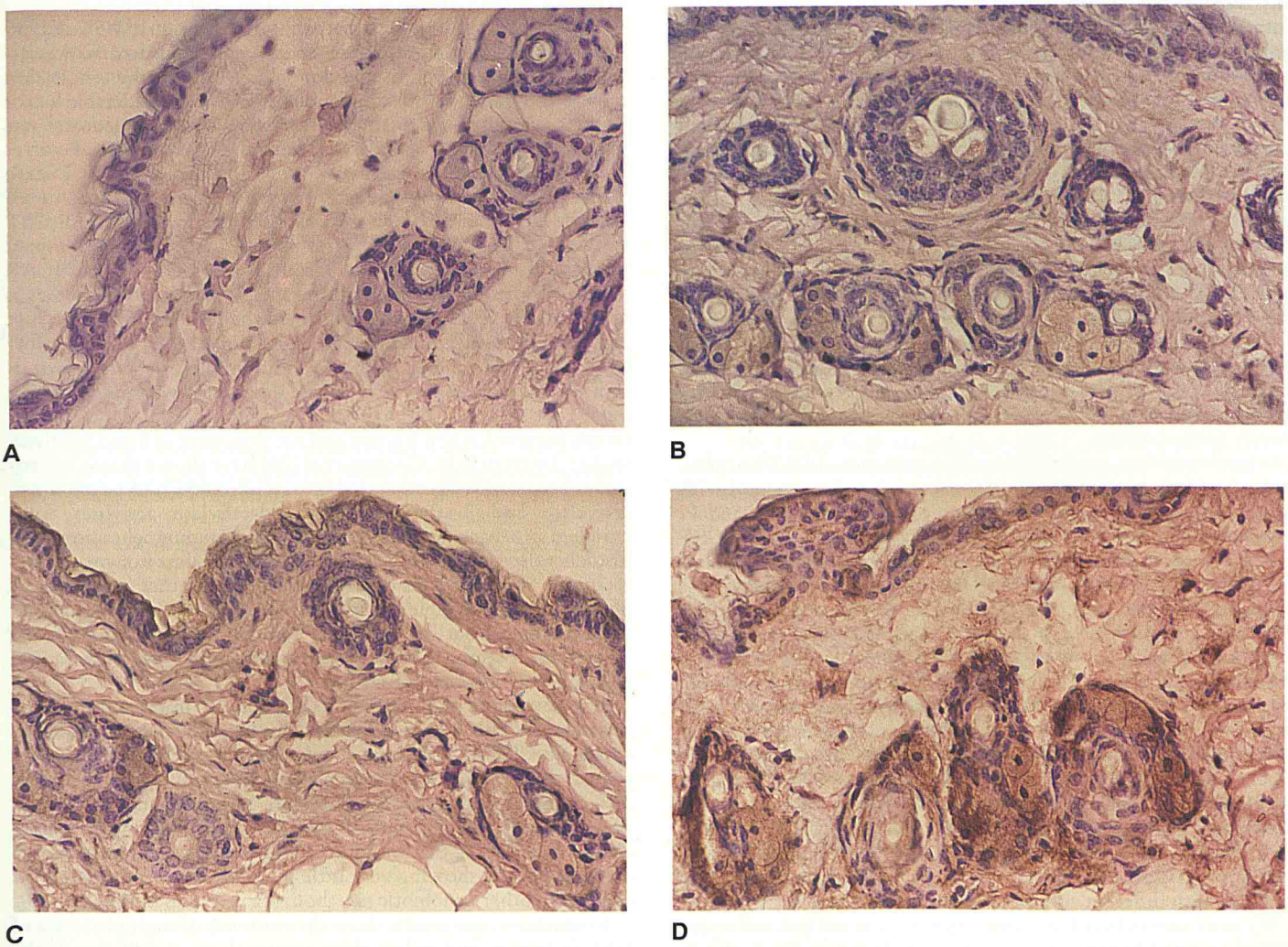

Figure 3. In situ immunohistochemical localization of GST in SENCAR mouse skin. Paraffin-embedded sections from the skin were deparaffinized, incubated with 1 : 100 dilution of antibodies to GST, and the color was visualized using avidin-biotin–conjugated peroxidase Vectastain ABC kit. *A*, negative control without antibody; *B*, sections probed with antibody to Alpha class of GST isozymes; *C*, sections probed with antibody to Mu class of GST isozymes; *D*, sections probed with antibody to Pi class of GST isozymes.

paraffin wax, and sequential 5-μm sections were cut. All sections were deparaffinized aand rehydrated. Endogenous peroxidase activity was blocked by immersing the slides in 30% (w/v) H_2O_2/methanol (1:2, v/v). The slides were then washed with PBS (10 mM potassium phosphate containing 0.9% NaCl, pH 7.4) and incubated with Vectastain blocking solution for 30 min at 25°C in a humidity chamber. The blocking solution was replaced with the primary rabbit antibody to GST class Alpha, Mu, and Pi (diluted 1:100 in PBS containing 0.1% BSA) isozymes. The rest of the procedure for visualization of antibody reacting regions was performed by using the Vectastain avidin-biotin peroxidase method with diaminobenzidine as peroxidase substrate, essentially as described in vendors protocols. In control situations, the primary antibody was omitted and the processing was the same as described above. Cellular nuclei were stained with hematoxylin to give a blue contrast visualization against the brown antibody-reacting region.

RESULTS AND DISCUSSION

Table I compares GST activity in human and rodent skin cytosol with a number of substrates. CDNB was found to be the most efficient substrate for both rodent and human skin cytosolic GST. However, human skin cytosol showed relatively lower activity towards CDNB as compared to rodent skin cytosol. Conversion of LTA_4 to LTC_4, a GST-dependent metabolism of this physiologically important substrate [25–27] in various tissues, was at a maximum with rat skin cytosol, followed by mouse and human skin cytosol. Ethacrynic acid was preferentially metabolized by mouse skin cytosol, whereas with rat and human skin cytosol enzyme activity was relatively low. On the other hand, GSH-peroxidase activity towards cumene hydroperoxide and bromosulfophthalein conjugation with GSH were not detectable in rodent and human skin cytosol. Styrene 7,8-oxide and 4,5-BPO were also found to be conjugated with GSH in the presence of skin cytosol from all three species. These results indicate a selective preference of GST for different substrates in cutaneous tissue, suggesting the possibility of the existence of multiple isozymes and species-specific expression in the skin. It has not been possible to classify GST isozymes based solely on their substrate specificity because of overlapping catalysis of the substrates with various isozymes. However, Mannervik [2] used this property as one means of classification, showing that the Alpha class contains isozymes with high GSH peroxidase activity towards cumene hydroperoxide whereas the Mu family has high activity with epoxides.

In further studies, we investigated the existence of GST isozymes in skin based on specific immunocrossreactivity of the cytosol with the monospecific polyclonal antibody to the Alpha, Mu, and Pi classes of GST isozymes. Data shown in Fig 1 indicate that the GST Pi is the predominant isozyme in all three species (Fig 1*C, lanes 1–3*). The Alpha class of isozymes (Fig 1*A*), on the other hand, was expressed in human skin *(lane 1)*, whereas the lack of specific reactivity with rodent skin *(lanes 2* and *3)* suggested either the absence of this class of enzymes or its presence below the level of detection. Western blot analysis of rodent and human skin cytosol with the Mu class of GST antibody (Fig 1*B*) showed immunoreactivity with rat and mouse cytosol *(lanes 2, 3)*, whereas no detectable reaction with human skin cytosol *(lane 1)* was observed. These data provide direct evidence for the existence of multiple forms of GST in rodent and human skin. Our data are in agreement with a recent report that human skin possesses GST belonging to the Alpha and Pi classes of isozymes [12] and is devoid of the Mu class of GST. This species-specific expression may be relevant to their variation in catalytic activities with different substrates as shown in Table I.

We next assessed GST isozyme expression in normal and transformed cultured human keratinocytes. As shown in Fig 2, the Pi class of isozyme was abundant in normal keratinocytes (KER) and in two squamous cell carcinoma cell lines (SCC-4 and SCC-13). In each of these surface epithelial cells, small amounts of Alpha isozyme(s) was found to be present, whereas the Mu class of isozyme(s) was not detectable (data not shown).

Table IV. Age-related change in Rat Skin Cytosolic GST Activity with CDNB as a Substrate[a]

Age (days)	GST Activity (nmol conjugate/min/mg protein)
5	48.32 ± 3.85
12	49.89 ± 2.72
19	55.65 ± 4.30
25	60.43 ± 4.55
32	64.59 ± 5.23
67	65.72 ± 5.85

[a] Skin from 15,8,4,2,2 and 2 male rats was pooled for the determination of the activity at 5,12,19,25,32, and 67 days of age, respectively. Five-d-old rats did not require any shaving; other animals were shaved using electric clippers. Skin cytosol was prepared and GST activity using CDNB as a substrate was determined as described in *Materials and Methods*. Each value represents represents the mean ± SD of three to four determinations.

Subcellular localization of GST activity in skin cytosol was investigated. Because CDNB is the conventional substrate and, as shown by data in Table I, it was the most efficient substrate for cutaneous GST, we employed CDNB for these studies. As shown in Table II, the maximum GST activity occurred in the cytosolic fraction. Enzyme activity in the microsomal pellet was less than 10% of that in the cytosol. Western blot analysis also confirmed the predominant existence of GST enzymes in cytosol (data not shown).

In additional studies, the topographic localization of GST activity in two major skin compartments, epidermis and dermis, was assessed. Because epidermal and dermal layers from the whole skin cannot be separated from adult animals without considerable loss of enzyme activities, these studies were conducted in neonatal rats where the separation of the two layers can be accomplished conveniently by immersing the skin in 10 mM dithiothreitol [15]. Data in Table III show that enzyme activity in the dermal layer was higher than in the epidermis. We next assessed the localization of GST activity across the skin using immunohistochemical techniques. Immunohistochemical in situ localization of GST in SENCAR mouse skin using the Alpha, Mu, and Pi classes of antibodies is shown in Fig 3. These data confirmed the results of Western blot analysis indicating a predominant expression of the Pi isozyme followed by the Mu and no Alpha. In addition, these data show that in skin GST is predominantly localized in sebaceous glands and in the outer root sheath of the hair follicles (Fig 3). Similar localization trends occurred when human and rat skin were utilized for these studies. Interestingly, Coomes et al [28] have shown that in microsomes prepared from untreated murine skin aryl hydrocarbon hydroxylase and 7-ethoxycoumarin-0-deethylase activities were enriched in sebaceous cells. Using enzyme immunochemistry and histochemistry employing antibodies raised against isozymes of cytochrome P-450, Baron et al [29] have shown that antibodies to phenobarbital-inducible hepatic cytochrome P-450 stain epidermal cells, the outer root sheath of hair follicles, and sebaceous glands. Therefore, it seems that the localization of GST and cytochrome P-450 in the skin may be similar.

The developmental pattern of GST activity in rat skin was followed with CDNB as the substrate. As shown by data in Table IV, cutaneous GST activity showed only a moderate change during the postnatal development in rat. In 32-d-old rat skin, GST activity was 34% higher than that in 5-d-old rat skin. These data suggest that the skin of even very young animals is capable of detoxifying xenobiotics as efficiently as that of adults. This is in agreement with our previous finding showing very little postnatal development-related changes in other xenobiotic metabolizing enzymes in the skin [30].

In summary, our results show the existence of multiple forms of GST in human and rodent skin; among these, the Pi class is the predominant isozyme.

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